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Identification of White Adipocyte Progenitor Cells In Vivo

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SUMMARY

The increased white adipose tissue (WAT) mass associated with obesity is the result of both hyperplasia and hypertrophy of adipocytes. However, the mechanisms controlling adipocyte number are unknown in part because the identity of the physiological adipocyte progenitor cells has not been defined in vivo. In this report, we employ a variety of approaches, including a noninvasive assay for following fat mass reconstitution in vivo, to identify a subpopulation of early adipocyte progenitor cells (Lin^-:$CD29^+$:$CD34^+$:$Sca\text{-}1^+$:$CD24^+$) resident in adult WAT. When injected into the residual fat pads of A-Zip lipodystrophic mice, these cells reconstitute a normal WAT depot and rescue the diabetic phenotype that develops in these animals. This report provides the identification of an undifferentiated adipocyte precursor subpopulation resident within the adipose tissue stroma that is capable of proliferating and differentiating into an adipose depot in vivo.

INTRODUCTION

Obesity, the excessive accumulation of white adipose tissue (WAT), is a highly prevalent condition that results from an imbalance of energy expenditure versus food intake, leading to an increase in the storage of energy in the form of lipid within WAT (Rosen and Spiegelman, 2006). Obesity is associated with increased risk of type II diabetes, hyperlipidemia, hypertension, and certain types of cancer, causing a major public health problem (Kopelman, 2000). Despite the clinical importance of obesity, our understanding WAT development in vivo is extremely limited. The increase in adipose mass in obesity is the result of both hypertrophy, an increase in adipocyte size, and hyperplasia, an increase in cell number (Hirsch and Batchelor, 1976; Lane and Tang, 2005). Since mature adipocytes are postmitotic (Simon, 1965), adipocyte hyperplasia in adults requires that new adipocytes be produced from the differentiation of precursor cells. However, the identity of the precursor cells in vivo is unknown (Gesta et al., 2007), and the cellular and molecular mechanisms regulating adipogenesis in vivo are poorly understood.

In vitro studies using well-characterized preadipocyte cell lines have elucidated a transcriptional cascade that is required for adipogenesis, involving the sequential induction of a series of transcription factors, including Krox20, Klf4, C/EBPδ, C/EBPβ, C/EBPα, and PPARγ, that leads to the formation of differentiated, lipid-laden adipocytes (Birsoy et al., 2008a; Gonzalez, 2005). Experiments studying the ability of preadipocyte cell lines to differentiate into adipocytes have shown that these cell lines have a limited ability to differentiate in vivo (Fischbach et al., 2004; Mandrup et al., 1997). Previous reports have also shown that cells derived from the WAT stromal-vascular fraction (SVF) from mice and humans can differentiate into several lineages, including adipocytes, in primary cell culture (Zheng et al., 2006; Zuk et al., 2001), and these multipotent cells have been named adipocyte-derived stem cells (ADSCs). However, the capacity of ADSCs and primary preadipocytes to differentiate into adipocytes in vivo is limited (Hemmrich et al., 2005; Hong et al., 2006; Patrick et al., 1999; Sengenes et al., 2005). Thus, the identity of the in vivo adipocyte precursor cells is unknown.

Here, we report the use of fluorescence-activated cell sorting (FACS) in combination with a functional in vivo transplantation scheme to identify early adipocyte precursor cells resident in the WAT vascular stroma. Analogous assays employing precursor cell transplantation in vivo have previously been developed for the study of hematopoietic, mammary epithelial, and spermatogonial stem cells (Brinster and Zimmermann, 1994; Deome et al., 1959; Ford et al., 1956; Shackleton et al., 2006; Spangrude et al., 1988). We assayed the adipogenic potential of different stromal cell populations in A-Zip lipodystrophic transgenic mice, which have a generalized lack of adipose tissue, and show that the residual WAT depots of A-Zip mice can provide a microenvironment that supports proliferation and differentiation of adipose tissue from a small subpopulation of cells isolated from wild-type WAT stroma.

RESULTS

FACS Isolation of Adipogenic Cells Expressing Stem Cell Markers in the Stroma of White Adipose Tissue

WAT is composed of multiple cell types, including adipocytes, vascular cells, and populations of fibroblast like cells, that have not been well characterized (Cinti, 2005). So that potential adipocyte progenitor cells could be identified, the SVF of wild-type WAT was fractionated (Rodbell, 1964) and FACS was performed

for the isolation of distinct cell populations on the basis of the differential expression of previously characterized cell-surface markers expressed by stem cell populations in other tissues.

Populations of individual cells were first sorted on the basis of their expression of CD29 (β1-integrin) and CD34, both of which are expressed by several stem cell populations (Shackleton et al., 2006; Trempus et al., 2003; Wilson et al., 2007). Four populations were isolated: $CD29^{-}$:$CD34^{-}$ (P1), $CD29^{lo}$:$CD34^{-}$ (P2), $CD29^{lo}$:$CD34^{+}$ (P3), and $CD29^{hi}$:$CD34^{+}$ (P4). Sorted cells were plated and grown to confluence, and the adipogenic potential of these sorted populations (P1–P4) was analyzed in vitro by oil red O staining. The induction of adipocyte differentiation was tested through the use of a standard protocol for adipogenesis that employs a cocktail of insulin, glucocorticoids, and isobutylmethylxanthine (IBMX) (see the Experimental Procedures). Only the P3 fraction of $CD29^{hi}$:$CD34^{+}$ cells showed significant differentiation in vitro, with accumulation of cells displaying unilocular lipid droplets (Figure S1 available online).

Having established that there was a subpopulation of cells in adipose stroma that was capable of differentiating into adipocytes in vitro, we next performed FACS for additional cell surface to further purify the adipogenic subpopulations. The strategy that was used in these studies is shown (see Figure 1) and described below. First, endothelial cells and hematopoietic cells were depleted from the SVF on the basis of staining of CD31, CD45, and Ter119, generating a lineage-negative (Lin^{-}) population. This step removed ~25% of the cells in the SVF. Second, the cell populations depleted of the lineage markers were separated on the basis of staining for CD34 and CD29, as described above. The Lin^{-}:$CD34^{+}$:$CD29^{+}$ subpopulation was further separated on the basis of staining for stem cell antigen 1 (Sca-1) and CD24 (Figure 1 and Figure 2A), two cell-surface proteins that are expressed on stem cell populations from other tissues (Shackleton et al., 2006; Spangrude et al., 1988; Wilson et al., 2007). The Lin^{-}:$CD29^{+}$:$CD34^{+}$:$Sca\text{-}1^{+}$ ($Sca\text{-}1^{+}$) population comprises approximately half of the cells present in the SVF (53.5%), but only a small population of cells also expressed CD24 with the total number of Lin^{-}:$CD29^{+}$:$CD34^{+}$:$Sca\text{-}1^{+}$:$CD24^{+}$ ($CD24^{+}$) cells constituting in total ~0.08% of the cells in the SVF (see Figure 1). The $Sca\text{-}1^{+}$ population was negative for CD105 (endoglin) and CD117 (c-kit) (data not shown), distinguishing these cells from bone marrow-derived mesenchymal stem cells and hematopoietic stem cells (Li and Johnson, 1995; Sun et al., 2003).

We next tested whether the $CD24^{+}$, Lin^{-}:$CD29^{+}$:$CD34^{+}$:$Sca\text{-}1^{+}$:$CD24^{-}$ ($CD24^{-}$) and Lin^{-}:$CD29^{+}$:$CD34^{-}$ ($CD34^{-}$) subpopulations can differentiate into adipocytes in vitro via the aforementioned protocol. We found that after exposure to an adipogenic cocktail in vitro, both the $CD24^{+}$ and $CD24^{-}$ populations developed into adipocytes with large, unilocular lipid droplets that stain with oil red O (Figure 2B). Similar levels of differentiation of both the $CD24^{+}$ and $CD24^{-}$ subpopulations were also observed when insulin alone was substituted for the full adipogenic hormone cocktail (data not shown). RT-PCR analysis of mRNAs derived from these differentiated adipocytes showed an approximately 7-fold increase in the expression of aP2 in both the $CD24^{+}$ and $CD24^{-}$ cultures versus the undifferentiated populations. Expression of Adipsin was increased 5-fold in the $CD24^{-}$ population and 25-fold in the $CD24^{+}$ population

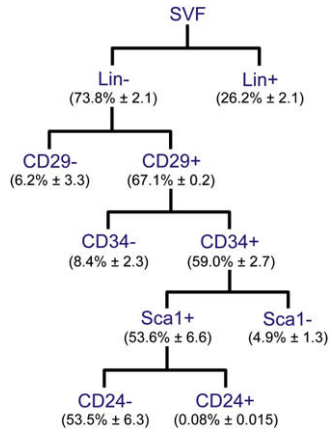

Figure 1. A Schematic Representation of the FACS Strategy

The scheme for the FACS analysis is shown as a dendrogram of the hierarchy developed for cell sorting. The percentage yield of each population compared to the starting number live single cells derived from the SVF is indicated. Results are shown as mean ± SD (n = 4).

(Figure 2C). The 5-fold greater induction of Adipsin expression in the $CD24^{+}$ versus $CD24^{-}$ adipocytes suggested that these are functionally distinct populations (confirmed in vivo, see below). Although both $CD24^{-}$ and $CD24^{+}$ populations could robustly differentiate into adipocytes, the $CD34^{-}$ cells failed to differentiate in response to the same adipogenic cocktail. Further experiments showed that the $CD24^{-}$ and $CD24^{+}$ cell populations are also capable of differentiating into bone, cartilage, and muscle in vitro (Figure S2). In addition, although the unfractionated SVF showed some ability to differentiate into adipocytes, it did so poorly when compared to the $CD24^{-}$ and $CD24^{+}$ populations, displaying significantly smaller numbers of oil red O positive cells with multilocular lipid accumulation (Figure 2B) and lower levels of Adipsin and aP2 expression (Figure 2C). The finding that the SVF is poorly adipogenic in comparison to the $CD24^{-}$ population, which represents 53.5% of the SVF, suggested that there may be inhibitory factors present in the unfractionated SVF. Quantification of the number of oil red O-positive cells derived from each cultured cell population isolated in the FACS sorting scheme revealed that the $CD24^{-}$ and $CD24^{+}$ were 24-fold and 19-fold enriched, respectively, for adipocytes versus the unfractionated SVF (Table S1). The extent of differentiation of the SVF in these experiments was similar to that documented in previous studies (Boquest et al., 2005; Izadpanah et al., 2006; Wagner et al., 2005). These data indicated that the FACS strategy that was employed enriched for adipocyte precursor cells.

Markers of Adipogenesis Are Induced in Lipodystrophic White Adipose Tissue

We next tested whether the different stromal cell fractions could reconstitute WAT in vivo by transplanting ~50,000 donor cells into recipient animals. However, we did not observe any differentiation of either the $CD24^{+}$ or $CD24^{-}$ subpopulation into adipocytes after injection of ~50,000 cells into adipose tissue of wild-type mice. This suggested that the microenvironment

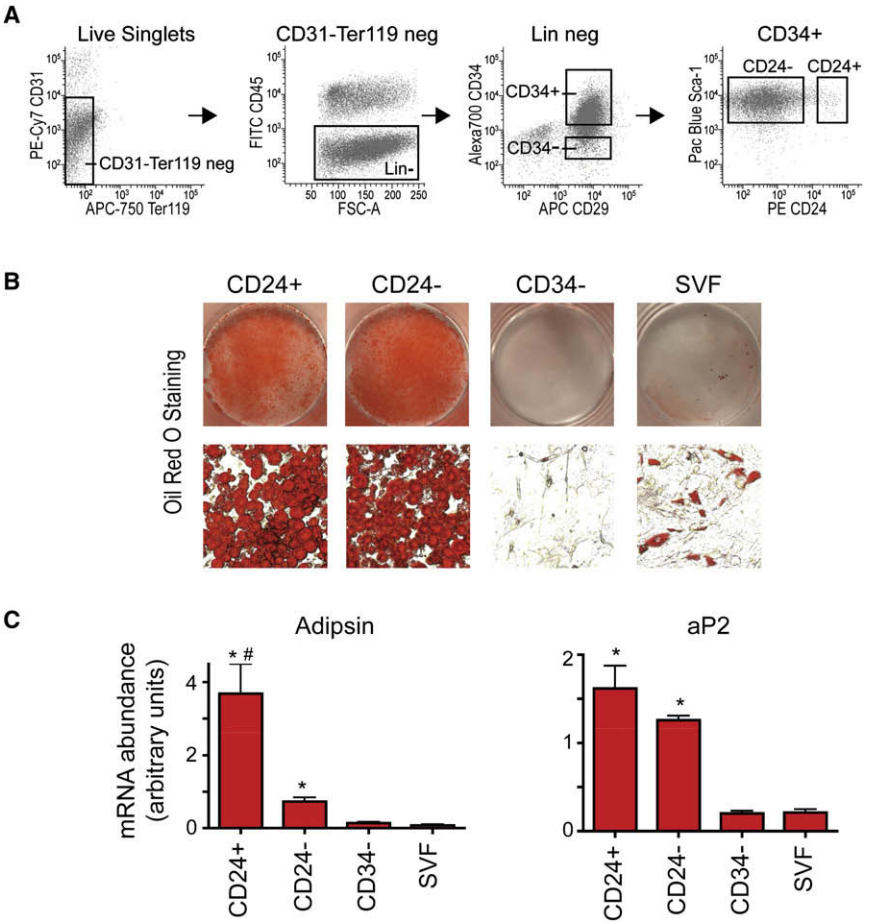

Figure 2. Identification of Adipogenic Cell Populations in Adipose Stroma

(A) Dot plots showing FACS staining profiles and gating (black boxes) of adipose SVF from 8-week-old wild-type mice. The live, PI-negative singlets were sorted for lack of CD31 and Ter119 expression (left-hand plot) and were further separated on the basis of expression of CD45 (second plot from the left). $CD45^-$ cells (Lin^-) were then sorted on the basis of expression of CD34 and CD29 (second plot from the right). $CD34^-$ cells were collected for analysis, and the $CD34^+$ cells were sorted on the basis of staining for Sca-1 and CD24 (right-hand plot). Representative $CD24^-$ and $CD24^+$ gates used for sorting are indicated.

(B) The sorted $CD24^+$, $CD24^-$, and $CD34^-$ cells and unsorted SVF were cultured and induced to differentiate with IBMX, glucocorticoid, and insulin. The cells were then cultured for 12 days prior to staining with oil red O. Macroscopic (upper panel) and microscopic (lower panel) views of the oil red O-stained dishes are shown (upper panel).

(C) RNA was isolated from cells differentiated for 12 days, and Taqman RT-PCR analysis was performed for the adipocyte markers Adipsin and aP2 on the indicated populations: $CD24^+$, $CD24^-$, $CD34^-$, and unsorted SVF. Results are shown as mean ± SEM for each population (n = 3). *, $p < 0.05$ between $CD24^+$ and $CD24^-$ versus $CD34^-$ and SVF. #, $p < 0.05$ between $CD24^+$ versus $CD24^-$.

of an adult adipose depot might not be conducive for the proliferation and differentiation of adipocyte precursor cells.

We next considered the possibility that A-Zip lipodystrophic mice could support adipogenesis after injection of the precursor cells capable of differentiating into adipocytes in vitro. A-Zip mice express a dominant-negative transcription factor (A-Zip), which inhibits C/EBP transcription factors, under the control of the aP2 promoter (Moitra et al., 1998). Since aP2 is expressed relatively late in adipogenesis, we reasoned that there might be an expansion of precursor cells upstream of the block in differentiation caused by the expression of the transgene.

Consistent with this hypothesis, A-Zip mice, which have a severe, generalized lack of WAT (Moitra et al., 1998), do retain small WAT depots discernable at the sites where mature WAT depots typically develop in wild-type animals (Figure 3A). Despite the paucity of adipocytes in A-Zip WAT, these small depots do contain a limited number of lipid-filled cells that are similar in size to wild-type adipocytes (Figure 3B), suggesting that some cells may escape the block in adipogenesis caused by A-Zip expression. There is also a marked increase in the number of stromal cells present in A-Zip WAT, and approximately half of this expanded cell population expresses the preadipocyte marker Pref-1 (Figures 3B and 3E).

We hypothesized that inhibition of the formation of mature adipocytes in the WAT depots of the A-Zip animals would lead to an increase of proliferation and differentiation of precursor cells upstream of the block in differentiation. If true, cells in the stromal fraction of these depots should continue to express genes induced early during adipogenesis (upstream of the block induced by the transgene) and continually proliferate in a manner similar to the clonal expansion phase of adipogenesis in preadipocyte cells lines induced to differentiate in vitro. To test this possibility, we analyzed the expression of genes typically induced early during adipogenesis in vitro in the A-Zip WAT depots. We found that the RNA levels for the early markers of adipogenesis, Klf4, Krox20, and C/EBPδ, were increased in A-Zip WAT depots relative to wild-type WAT by 191%, 498%, and 291%, respectively, whereas the expression of PPARγ, a marker of mature adipocytes, was decreased to 56% of the levels in wild-type tissue (Figure 3C). Immunohistochemistry for the preadipocyte marker Pref-1 demonstrates both the presence of preadipocytes in wild-type WAT and an increase in Pref-1-positive cells in lipodystrophic WAT (Figures 3B and 3E). Immunohistochemistry for Krox20 and Klf4 indicated very low expression of these factors at the protein level in wild-type WAT (Figures 3D and 3E), with a significant increase in the numbers of Klf4- and Krox20-positive cells in A-Zip WAT (Figures 3D and 3E). In lipodystrophic WAT, there was also a similar increase in the level of CREB phosphorylation, one of the earliest events observed during adipogenesis in vitro (Zhang et al., 2004).

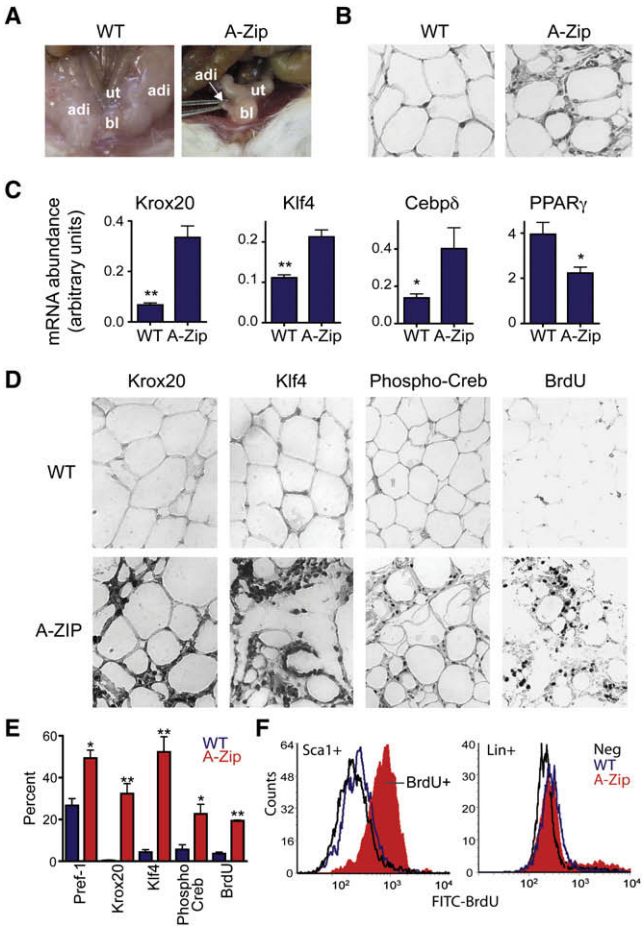

Figure 3. A-Zip Lipodystrophic Adipose Tissue Provides an Adipogenic Microenvironment

(A) Ventral view of the lower abdomen of female littermate wild-type (WT) and A-Zip mice at 12 weeks of age. The bladder (bl), uterus and fallopian tubes (ut), and parametrial WAT (adi) are indicated.

(B) Results from immunohistochemistry for Pref-1 in WT and A-Zip mice are shown.

(C) Taqman RT-PCR was performed for the markers of early adipogenesis, Krox20, Klf4, and Cebpδ, as well as PPARγ. RNA was isolated from adult A-Zip and WT parametrial adipose tissue. Results are shown as mean ± SEM, n = 3 (*, $p < 0.05$; **, $p < 0.001$).

(D) Immunostaining of parametrial WAT from A-Zip and WT mice for the early markers of adipogenesis Krox20, Klf4, and phospho-CREB, as well as the proliferation marker BrdU, is shown. For BrdU incorporation analysis, mice were treated with BrdU for 7 days prior to immunostaining.

(E) Quantification of the immunostaining results shown as the percent of cells positive for the indicated immunohistochemical marker. Data are shown as mean ± SEM. n = 125–614 cells (*, $p < 0.05$; **, $p < 0.001$).

(F) Flow cytometry analysis of BrdU incorporation. Wild-type (WT), A-Zip, and an A-Zip isotype control (Neg) are shown for both the Sca-1$^+$ adipogenic cell population (left panel) and the Lin$^+$ population (right panel), demonstrating the proliferative nature of the Sca-1$^+$ cells from lipodystrophic mice.

During adipogenesis in vitro, preadipocytes proliferate during a stage of clonal expansion (Bernlohr et al., 1985) that coincides with the expression of the aforementioned transcription factors. To test whether proliferation is also increased in lipodystrophic WAT, we treated A-Zip mice with BrdU for 7 days. Analysis of BrdU incorporation demonstrated that A-Zip adipose tissue contains large numbers of proliferating cells compared to wild-type adipose, in which there are low levels of BrdU incorporation (Figures 3D and 3E). Flow cytometry demonstrated that the cells from A-Zip mice that incorporated BrdU overlapped substantially with the adipogenic Sca-1$^+$ cell subpopulation (which includes both the CD24$^+$ and CD24$^-$ subpopulations) that we showed are capable of robustly differentiating into adipose tissue in vitro. In contrast, the Sca-1$^+$ population from wild-type animals did not incorporate BrdU (Figure 3F). The increase in BrdU incorporation in A-Zip mice is almost exclusively due to proliferation of the Sca-1$^+$ population because BrdU incorporation into the Lin$^+$ population was not different between cell populations derived from wild-type and A-Zip WAT (Figure 3F). Of note, the number of CD24$^+$ cells is increased 277% in A-Zip WAT (Figure S3).

Reconstitution of Functional White Adipose Tissue In Vivo

These data suggested that the adipose tissue depot of lipodystrophic mice might provide a microenvironment, or niche, capable of supporting the proliferation and differentiation of adipocyte precursor cells. We next tested whether the FACS-sorted subpopulations could develop into adipose tissue when injected into the WAT depots of A-Zip mice. We used cells derived from *ubiquitin-GFP* mice, which express GFP ubiquitously, as donors in transplantation experiments in which a total of 50,000 freshly purified cells from the CD24$^+$, CD24$^-$, or CD34$^-$ subpopulations were injected unilaterally into the parametrial WAT depot of A-Zip recipients.

Inspection of the injected depots 12 weeks after injection revealed that injection of CD24$^+$ donor cells developed into a normal-sized WAT depot at the site of injection (Figure 4A). Reconstitution of the injected parametrial fat pad was seen in 11 out of 20 animals that received the injection of CD24$^+$ donor cells, which is comparable to the success rates achieved in similar grafting experiments from mammary gland (~80%) and testis (18%–37%) (Brinster and Zimmermann, 1994; Smith and Medina, 1988). The depots that develop after injection of 50,000 CD24$^+$ cells were composed almost entirely of GFP-positive (GFP$^+$) cells with the unilocular morphology characteristic of mature white adipocytes (Figure 4B), confirming that the adipose depot that developed was derived from the donor cells. In fact, the reconstituted WAT appeared to be identical to the *ubiquitin-GFP* donor WAT with the exception of the vasculature, which was GFP$^+$ in the donor WAT but GFP-negative in the reconstituted tissue (Figure 4B). To further investigate the contribution of the CD24$^+$ cells to other cell populations within the reconstituted WAT, we analyzed cell subpopulations for GFP expression by FACS. Although GFP$^+$ donor cells remained present in the CD24$^+$ population in the reconstituted WAT (15.6% ± 1.3%), GFP was not detected in the endothelial (CD31$^+$) or hematopoietic populations (CD45$^+$) (Figure 4C), indicating that the CD24$^+$ cells populations do not contribute to these lineages in the reconstituted WAT depots.

Injection of the unfractionated SVF resulted in a low success rate of WAT reconstitution (1 of 10 recipients), and the Lin$^-$: CD34$^+$ population, which also contains the CD24$^+$ population,

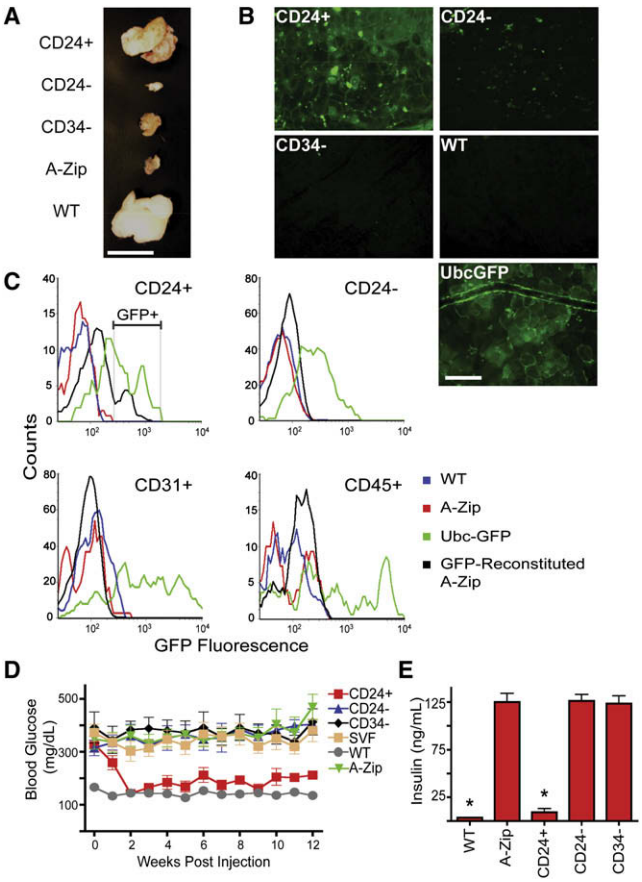

Figure 4. The CD24$^+$ Cell Population Develops into Functional White Adipose Tissue

(A) Cells sorted from the CD24$^+$, CD24$^-$, or CD34$^-$ populations were injected into parametrial adipose tissue pads of A-Zip mice. The fat pads were removed from these animals and an uninjected A-Zip control and were compared to a wild-type (WT) parametrial fat pad (bottom image). The scale bar represents 1 cm.

(B) Whole-mount fluorescent imaging for GFP is shown for A-Zip fat pads that were injected with CD24$^+$, CD24$^-$, and CD34$^-$ cells with WT and *ubiquitin-GFP* donor WAT (UbcGFP) controls. The scale bar represents 100 μM.

(C) FACS analysis of GFP expression in the SVF of reconstituted WAT. Histograms are shown for different SVF subpopulations, as indicated: CD24$^+$, CD24$^-$ Ter119$^-$:CD31$^+$ (CD31$^+$), and Ter119$^-$:CD45$^+$ (CD45$^+$).

(D) Plasma (glucose) from A-Zip mice injected with the CD24$^+$, CD24$^-$, CD34$^-$, or SVF cells, as well as WT and A-Zip controls. Results are shown as mean ± SEM. Correction of blood glucose by the CD24$^+$ population is significant ($p < 0.05$) at week 2 and remains significant through the 12 week experimental endpoint ($n = 5$). Results are shown as mean ± SEM.

(E) The plasma (insulin) at the 12-week endpoint of A-Zip mice that were injected with the CD24$^+$, CD24$^-$, or CD34$^-$ cell populations and noninjected wild-type and A-Zip controls is shown. Results are shown as mean ± SEM; *, $p < 0.05$ ($n = 5$).

also failed to reconstitute WAT (zero of six injections), indicating that the CD24$^+$ population is highly enriched for adipogenic precursor cells. The CD24$^-$ (zero of ten recipients) and CD34$^-$ (zero of six recipients) populations also failed to reconstitute WAT after injection into A-Zip mice. Small numbers of GFP$^+$ cells were detectable in adipose depots injected with the CD24$^-$ cells, indicating that these cells were able to engraft in WAT. However, in this case, GFP$^+$ lipid-filled cells were not detected, suggesting that these cells have a limited ability to differentiate. Overall, these studies showed that the CD24$^+$ cells were the only subpopulation of the SVF capable of forming an adipose tissue depot. (See Table S2 for a summary of the fate of all of the injected cell populations.) As mentioned previously, injection of the CD24$^+$ population into wild-type fat pads did not lead to the appearance of GFP-labeled adipocytes, suggesting that the microenvironment of the A-Zip adipose depot is necessary for the differentiation of the precursor cells (data not shown).

We next tested whether the adipose depots that formed in diabetic A-Zip mice after injection of 50,000 CD24$^+$ donor cells were functional. Plasma glucose and insulin levels in A-Zip mice injected with the CD24$^+$, CD24$^-$, or CD34$^-$ cell populations were compared to noninjected A-Zip and wild-type control animals. Blood glucose was normalized after injection of the CD24$^+$ population into the A-Zip animals in those animals where an adipose depot had formed (Figure 4D). None of the other cell populations showed this capacity. The decrease of blood glucose levels was significant within 2 weeks of injection and was sustained for 12 weeks, at which point the animals were sacrificed and the adipose depot was analyzed for assessment of whether the precursor cells had engrafted. Plasma insulin levels were also normalized in these CD24$^+$-injected animals (Figure 4E). In addition, the plasma levels of adiponectin, a hormone that is secreted specifically from mature adipocytes (Hu et al., 1996), is also increased in the mice that display reconstituted WAT versus the baseline levels of A-Zip controls (10.2-fold, $p < 0.05$) (Figure S4). These data indicate that the CD24$^+$ cells generate functional WAT when injected into the parametrial WAT depot of A-Zip mice.

One limitation of these experiments is that the formation of an adipose depot could not be assessed until the 12 week experiment had been completed. In order to monitor the ability of the CD24$^+$ cells to develop into adipocytes prior to necropsy, we made use of bacterial artificial chromosome (BAC) transgenic mice expressing luciferase under control of the leptin promoter (Birsoy et al., 2008b). The expression of luciferase in these mice is restricted to adipose tissue (Figure 5A) and to mature adipocytes within adipose tissue (Figure 5B). Luciferase activity can be followed noninvasively in vivo by imaging with a CCD camera. Since cells of the SVF do not express the luciferase reporter (Figure 5B), the transplant of donor cells from the *leptin-luciferase* BAC transgenic mice allows us to follow the development of mature adipocytes (as marked by luciferase activity) from the donor cells noninvasively in vivo.

SVF cells were isolated from *leptin-luciferase* mice, purified by FACS via the protocol described above, and injected in the fat depots of A-Zip mice. Mice injected with the CD24$^+$ cell population derived from *leptin-luciferase* animals expressed luciferase in the injected depot beginning at 2 weeks after injection (in three of six injected animals) with the expression of luciferase gradually increasing through the end of the 12 week time course (Figure 5C). In contrast, injection of the control CD34$^-$ cells, which do not differentiate in vitro (Figure 2B), did not yield detectable luciferase expression (Figure 5C). Transplantation of the CD24$^-$ population, which shows adipogenic potential in vitro, showed extremely low levels of luciferase expression in the

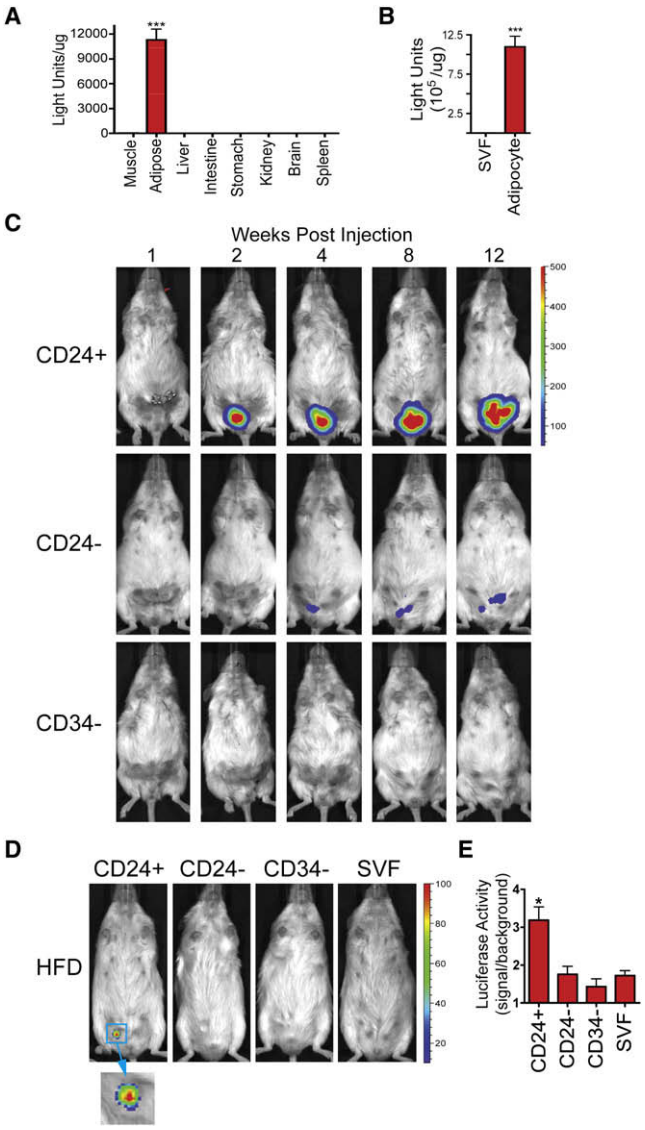

Figure 5. Imaging of Adipocyte Differentiation In Vivo

(A and B) Luciferase activity in tissues and fractionated WAT isolated from *leptin-luciferase* mice. The indicated tissues were isolated, and luciferase activity was determined. Data shown are normalized to total protein in each lysate. Results are shown as mean ± SEM; ***, $p < 0.001$ ($n = 3$).

(C) Luciferase imaging of adipocyte differentiation in the A-Zip transplantation model at indicated time points after injection of 50,000 donor cells from the $CD24^+$, $CD24^-$, or $CD34^-$ cell populations.

(D) Luciferase imaging of adipocyte differentiation in wild-type mice fed a high-fat diet. Wild-type mice were injected with the indicated cell populations, $CD24^+$, $CD24^-$, $CD34^-$, or SVF, allowed to recover for 2 weeks, and then treated with high-fat diet for 6 weeks prior to luciferase imaging. Luciferase signal was detected in three of eight animals injected with the $CD24^+$ population not detected in the $CD24^-$, $CD34^-$, or unfractionated SVF injected animals (zero of five for each population). A magnified image of the luciferase signal from the $CD24^+$ image is shown below the panel (blue border). The color bars indicate photon counts relative to pseudocolor for the adjacent panels images.

(E) Quantitation of luciferase activity from HFD-fed mice injected with FACS-sorted cell populations. Luciferase activity is significantly increased after injection of the $CD24^+$ cells versus the levels in animals receiving the grafts of other three stromal cell populations. Results are shown as mean ± SD; *, $p < 0.05$ ($n = 3$).

injected adipose depot after 1 month. This result suggested that the $CD24^-$ subpopulation might be capable of adipogenesis in vivo but that the differentiation or proliferative potential of these cells is limited. This observation is consistent with finding that the $CD24^-$ population was unable to correct blood glucose levels in the lipodystrophic mice (Figure 4D). None of the cell populations, including the $CD24^+$ cell population, expressed luciferase after injection into WAT of wild-type mice at any point in a 12 week time course (data not shown).

We next tested the fate of *leptin-luciferase*-derived FACS-sorted cells after injection into wild-type mice fed a high-fat diet (HFD). After injection of 50,000 $CD24^+$ cells into the fat pad of high-fat fed mice, there was clearly evident luciferase activity in three of eight injected animals, a frequency consistent with that seen after injection of precursor cells into lipodystrophic mice (Figures 5D and 5E). In contrast, we failed to detect luciferase activity in any of the five animals that received injections of an equivalent number of $CD24^-$, $CD34^-$, or SVF cells (Figures 5D and 5E). Although significant, the luciferase signal after injection of the $CD24^+$ cells was lower than that seen after injection of precursor cells into the A-Zip mice. Overall, these data indicate that the $CD24^+$ cell population also has the unique ability to differentiate into adipocytes in the context of diet-induced obesity as compared to the other stromal cell populations.

DISCUSSION

Obesity is associated with an increased adipocyte number (Faust et al., 1978; Wise, 1975). In addition, a recent study following C^{14} levels in WAT has confirmed that the adipocytes continuously turn over in adult humans (Spalding et al., 2008). These findings, coupled with the knowledge that mature adipocytes are postmitotic, strongly suggest that there is an adipose tissue precursor cell population resident in adipose tissue. However, studies of the regulation of WAT mass in vivo have been limited by the fact that physiologic adipocyte precursor cells have not been identified. As a consequence, our understanding of the developmental pathways by which adipose tissue forms and the mechanisms controlling the regulation of adipocyte number in vivo is limited.

In this report, we use FACS in combination with an assay for monitoring adipogenesis in vivo to identify a small subpopulation of $CD24^+$ cells in the adipose tissue stroma (~0.08% of total cells) that is capable of forming a normal-size fat depot in vivo and complementing the diabetic phenotype of lipodystrophic mice. These studies demonstrate that primary cells from the adipose tissue stromal fraction can proliferate and differentiate into functional, mature adipocytes to reconstitute a fully functional adipose depot in vivo. Precursor cells resident in WAT that are capable of reconstituting an adipose depot in vivo have not been previously identified. Immunohistochemical localization of CD24 in WAT demonstrated that $CD24^+$ cells are located perivascularly within WAT (data not shown), consistent with previous reports of the localization of putative adipocyte precursors (Cinti et al., 1984; Wassermann, 1926), a conclusion that has recently

been confirmed in vivo with PPARγ-reporter mice (Tang et al., 2008). The identification of these CD24$^+$ cells as adipocyte progenitors that are capable of adipogenesis in vivo now provides a potential means for identifying the cellular and molecular mechanisms regulating adipocyte number in vivo.

Although previous studies have shown that adipose tissue stromal cells can differentiate in vitro after several passages (Boquest et al., 2005; Izadpanah et al., 2006; Lee et al., 2004; Wagner et al., 2005), cells isolated in this manner were only able to produce fat pads in vivo when they were seeded into artificial scaffolds in vitro and induced to differentiate in culture, prior to implantation (Hemmrich et al., 2005; Patrick et al., 1999). In addition, the capacity of these cells to restore adipose tissue function was not assessed.

As an alternative, we employed FACS to separate cell populations from freshly isolated wild-type SVF on the basis of expression of a variety of well-characterized stem cell markers. This approach enabled us to identify two subpopulations of cells in wild-type WAT SVF with adipogenic potential in vitro (Figure 2). Previous studies have shown that cells derived from WAT SVF are capable of differentiation into multiple cell lineages, including adipocytes (Boquest et al., 2005; Izadpanah et al., 2006; Lee et al., 2004; Wagner et al., 2005). However, in numerous experiments, including ours, the differentiation of unfractionated SVF into adipocytes in vitro is limited with only a small percentage of cells showing multilocular lipid accumulation (Figure 2B). The CD24$^+$ and CD24$^-$ stromal subpopulations reported here both display robust adipogenesis in cell culture, with the majority of cells not only accumulating lipid but also developing a unilocular morphology with the induction of gene expression associated with mature adipocytes in vivo (Figures 2B and 2C). These results indicate that we have identified two subpopulations of WAT SVF that may be significantly enriched for adipocyte precursor cells compared to the unfractionated stromal fraction. Of note, the adipogenic CD24$^-$ population comprises approximately half of the unfractionated SVF, which differentiates poorly, suggesting that there may be inhibitors present in the SVF that are removed by purification of the Sca-1$^+$ cell populations. It is possible that the CD24$^-$ population is comprised of preadipocytes, a possibility supported by the expression of Pref-1 in these cells (data not shown). Also consistent with this hypothesis, luciferase expression indicated that the CD24$^-$ cells are capable of limited differentiation into adipocytes in vivo (Figure 5C), but they are unable to develop into a full-sized depot (Figure 4A).

To test the adipogenic potential of the SVF subpopulations identified in our studies in vivo, we developed a functional assay using A-Zip lipodystrophic mice as recipients. The use of A-Zip mice for these studies is important because they have a paucity of mature, functionally competent adipocytes (Moitra et al., 1998). In addition, WAT of A-Zip animals showed increased expression of early markers of adipocyte differentiation and high rates of proliferation, suggesting that it provides a niche conducive for promoting early events in adipogenesis (Figure 3). Indeed, we only observed adipose tissue development after injection of CD24$^+$ donor cells into A-Zip mice or HFD-fed animals. In contrast, we did not observe the development mature adipocytes after injection of an equivalent number of CD24$^+$ donor cells into the WAT of wild-type animals at weaning (data not shown). This result was not unexpected because, in contrast to the high rates of proliferation and differentiation in hematopoietic, epithelial, and spermatogonial stem cells previously used in analogous cell transplant studies (Bryder et al., 2006; Fata et al., 2001; Thayer et al., 2001), WAT in adult rodents displays only low levels of cell proliferation and new adipocyte formation (Greenwood and Hirsch, 1974).

As mentioned above, CD24$^+$ cells transplanted into mice fed a high-fat diet can also differentiate into adipocytes with the appearance of a significant level of luciferase activity 6 weeks after injection. None of the other stromal cell populations show luciferase activity after injection into the HFD-fed animals, showing that, as was also the case for transplants into lipodystrophic mice, the CD24$^+$ cells have unique developmental potential among the different populations in the adipose tissue SVF. However, in these experiments, the luciferase signal is significantly lower than that seen in the CD24$^+$-injected A-Zip animals (Figure 5). The lower signal in the HFD transplant study is likely due to a combination of factors, including competition between larger numbers of resident nonluciferase CD24$^+$ cells present within the wild-type adipose depot and the considerably smaller expansion of the adipose depot after transplantation into HFD mice as compared to the complete reconstitution of an adipose depot after injection of CD24$^+$ cells into a lipodystrophic adipose depot. The more limited contribution of the transplanted CD24$^+$ cells to the adipose depot in a wild-type setting is consistent with analogous studies of progenitor cells from other systems, such as the hematopoietic, spermatagonial, and mammary epithelial cells, where, in general, the native cell populations need to be ablated prior to transplantation in order for the transplanted cells to robustly repopulate the organ (Brinster and Zimmermann, 1994; Deome et al., 1959; Ford et al., 1956; Shackleton et al., 2006; Spangrude et al., 1988). The demonstration that the CD24$^+$ can contribute to the adipose depot after high-fat feeding but not in chow-fed animals suggests that the high-fat diet may create a state in which inductive factors promote adipocyte development, a possibility that is consistent with an increase in adipocyte number in adipose tissue after high-fat feeding (Johnson et al., 1971; Lemonnier, 1970, 1972).

The lack of normal mature adipose tissue in lipodystrophic animals leads to diabetes, also permitting a determination of the functional competence of the transplanted adipose tissue precursors. It has previously been shown that the transplant of 900 mg of normal adult WAT into A-Zip mice is required for correction of their insulin resistance and hyperglycemia (Gavrilova et al., 2000) and that transplants of smaller amounts of adipose tissue failed to correct diabetes in these animals. However, in our experiments, injection of only 50,000 CD24$^+$ cells was capable of reconstituting a normal-sized WAT depot and correcting the diabetes of a lipodystrophic animal. This is several logs lower than the number of cells required for correction of the diabetic phenotype after transplant of mature adipose tissue (Gavrilova et al., 2000) or that are present in the fully formed adipose depots that develop from these cells. These results strongly suggest that the CD24$^+$ donor cells proliferate prior to differentiating into mature adipocytes, suggesting that adipocyte progenitors with proliferative capacity are resident in the adult WAT SVF fraction.

Injections of 3H or BrdU into wild-type animals failed to label adipose tissue in previous studies, indicating that the proliferative potential of adult adipose tissue might be limited (Greenwood and Hirsch, 1974). Our finding that a subpopulation of SVF cells is able to proliferate to form a mature fat pad in A-Zip mice and contribute to adipocytes upon HFD treatment, along with recent findings that human adipose tissue turns over at a low rate (Spalding et al., 2008), suggests that cells resident in WAT do have significant proliferative capacity in vivo but that the proliferation and differentiation of these cells is tightly regulated in normal animals.

The lack of differentiation of any of the cell subpopulations upon injection into wild-type WAT suggests that the niche provided by A-Zip mice is critical for the expansion and differentiation of adipocyte progenitors. This possibility is consistent with the observation that intravenous injections of the $CD24^+$ cells into A-Zip mice did not result in the restoration of adipose tissue mass, luciferase expression, or correction of blood glucose levels (data not shown), indicating that these cells can not ''home'' to the adipose tissue in lipodystrophic mice. It is possible that the ability of lipodystrophic adipose tissue to support the proliferation and differentiation of precursor cells is at least partially the result of a loss of feedback inhibition of precursor cells by fully differentiated adipocytes. The observation that $CD24^+$ cells do not contribute to adipocyte populations after injection into wild-type WAT is also consistent with the possibility that the there may be inhibitory cell types and/or factors in normal adult adipose tissue. The nature of this inhibitory factor or cell type is unknown and is currently under investigation.

The identification of the $CD24^+$ cell subpopulation of adipose SVF as physiologic adipocyte precursors capable of producing functional WAT in vivo also provides a potential means for reconstitution of adipose depots in patients with lipodystrophy. For example, one could isolate an analogous cell population from human adipose depots as a source of donor cells for patients. Alternatively, since in many cases the genetic defect causing lipodystrophy is known, one could perform autologous transplants by isolating the adipose progenitor cells from affected patients and correcting the genetic defect ex vivo. Although the approach reported here for mice could be adapted to include other species, much additional investigation would be necessary for assessment of the potential of this approach for humans. Additionally, the finding that both the $CD24^-$ and $CD24^+$ cell populations are capable of differentiating into bone, cartilage, and muscle (Figure S2) suggests that these populations might also prove useful for the generation of other mesenchymal tissues.

The identification of an adipocyte precursor cell population provides a means for identifying tissue factors in wild-type and lipodystrophic WAT that regulate adipocyte proliferation and differentiation. Furthermore, the availability of the *leptin-luciferase* mice as donors in the A-Zip transplant assay, which allows the noninvasive monitoring of adipogenesis in vivo, should facilitate the identification of conditions that optimize engraftment of adipose tissue precursors. Overall, this approach will provide a means by which researchers can study the potential of cells from various sources to form WAT and creates new possibilities for the determination of the roles that individual factors play in the control and maintenance of WAT mass in vivo in a variety of nutritional states, including obesity.

EXPERIMENTAL PROCEDURES

Mice

FVBN/J *ubiquitin-GFP* mice were obtained from the Jackson Laboratory (Bar Harbor, ME). A-Zip mice have been described previously (Moitra et al., 1998) and were maintained by crossing of A-Zip male to FVBN/J female mice (Jackson Laboratory). The *leptin-luciferase* transgenic mice, originally generated in the C57Bl/6 mouse strain (Birsoy et al., 2008b), were reproduced by microinjection in the FVBJ/N, ensuring that donor cells would be isogenic to the recipient A-Zip mice. The specifics of the *leptin-luciferase* BAC used to make the FVB transgenic mice are as follows. The modified BAC contains ~22 kb of 5′ sequence and ~150 kb of 3′ sequence relative to the Leptin transcription start site, with the coding sequence of firefly luciferase inserted in the translation start site of Leptin. All animals were housed on a 12 hr light/dark cycle at 23°C with free access to water and food. All procedures were in accordance with the guidelines of The Rockefeller University Laboratory Animal Research Center. For high-fat-diet treatment, mice were fed 60% kcal fat rodent food (Research Diets, New Brunswick, NJ).

Antibodies

Antibodies were purchased from eBioscience unless otherwise stated, including the following: CD34-Alexa700, CD45-FITC, CD45-PE-Cy7, Terr-119-APC-Alexa750, CD31-PE-Cy7, CD24-PE, CD29-APC and Sca-1-Pacific Blue (Biolegend), Klf4 and Krox20 (Santa Cruz Biotech.), Pref1 (MLB), phospho-CREB (Cell Signaling Technologies), BrdU (Abcam), and Desmin (Chemicon). The CD24-biotin antibody used for immunohistochemistry was obtained from BD PharMingen.

Labeling, Analysis, and Sorting of Adipose Stromal-Vascular Cells

Subcutaneous and parametrial adipose tissue was excised and the SVF isolated as described (Rodbell, 1964). The isolated SVF was resuspended in ice-cold Dulbecco's modified Eagle's medium (DMEM) with 2% fetal bovine serum (FBS) for labeling. Antibody incubations were performed on ice for 15 min. Cells were washed and resuspended in Hank's balanced salt solution (HBSS) with 0.5 g/ml propidium iodide (Sigma-Aldrich) for sorting. BrdU incorporation was analyzed with the FITC BrdU Flow Kit (BD PharMingen) according to the manufacturer's protocol. Samples were sorted on a BD FACSAria cell sorter and analyzed on a BD LSRII (BD Biosciences) flow cytometer, each equipped with BD FACSDiva Software. For sorting, cells were initially selected by size, on the basis of forward scatter (FSC) and side scatter (SSC), followed by exclusion of dead cells on the basis of uptake of propidium iodide. Then, live cells were gated on both SSC and FSC singlets, ensuring that the staining of individual cells was analyzed. Next, the cells were separated on the basis of the cell-surface markers indicated.

Culture and Differentiation of Primary Cells

Freshly isolated cells were plated onto laminin-coated plates (BD Biosciences) in DMEM supplemented with 10% FBS (GIBCO) and 10 ng/mL bFGF (R&D Systems) and maintained in a 5% CO2 atmosphere. Cells were allowed to grow to confluence and were then held at confluence for 2 days without changing of the media prior to exposure to the differentiation cocktail (1 μg/ml insulin, 0.25 μg/ml dexamethasone, and 0.5 mM IBMX) in fresh media without addition of bFGF. After 72 hr of exposure to the differentiation cocktail, cells were maintained in DMEM with 10% FBS until day 12 for harvest. For staining, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 15 min and then rinsed in PBS, water, and 60% isopropanol sequentially. The cells were then stained with oil red O (0.7% in 60% isopropanol) for 10 min and rinsed with 60% isopropanol followed by water. For gene expression analysis, RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR was performed by the Taqman system (Applied Biosystems), with protocols and primers previously described (Birsoy et al., 2008a; Chen et al., 2005). Data from RT-PCR analysis is shown as mean ± SEM. Osteogenic and chondrogenic differentiation were performed as described

(Boquest et al., 2005). Alkaline phosphatase activity was determined with an alkaline phosphatase detection kit (Sigma-Aldrich). Myogenic differentiation was performed as described (Lee et al., 2005). In brief, the isolated SVF subfractions were mixed with myogenic C2C12 cells and then cultured in DMEM supplemented with 20% serum overnight. The media was then changed to DMEM with 1% FBS, and the cells were maintained for 5 days with media changes every other day. The cells were then fixed in paraformaldehyde, immunofluorescence was performed for desmin, and GFP fluorescence was visualized directly.

Analysis of WAT

For immunohistochemistry, parametrial adipose tissue was excised, fixed, and paraffin-embedded as described (Cinti et al., 2001). Immunohistochemistry was performed according to the VectaStain Elite ABC Kit protocols (Vector Laboratories). Antibody staining was visualized with the metal-enhanced DAB kit (Thermo Scientific). For immunohistochemical analysis of BrdU incorporation, antigen retrieval was done under pressure in a 2100 Retriever (PickCell Laboratories). Labeling of BrdU incorporation was done with the FITC BrdU Flow Kit (BD PharMingen).

In Vivo Adipogenesis Assay

Sorted cells from the Lin^-:$CD29^+$:$CD34^+$:$Sca\text{-}1^+$:$CD24^+$, Lin^-:$CD29^+$:$CD34^+$:$Sca\text{-}1^+$:$CD24^-$, and Lin^-:$CD29^+$:$CD34^-$ cell populations, or other populations indicated in Table S2, were injected directly into the presumptive parametrial adipose pad of 6- to 10-week-old A-Zip mice. Mice were anesthetized with isoflurane prior to performance of surgery for exposure of the parametrial (also known as reproductive or periuterine) WAT for injection. After the transplant surgery, mice were allowed to recover, and blood glucose levels were checked once weekly throughout the time course with the Ascensia Elite blood glucose monitoring system (Bayer Healthcare). Luciferase imaging was performed as described (Birsoy et al., 2008b) with an IVIS imaging system (Caliper Lifesciences). After 12 weeks, the mice were sacrificed, injected adipose tissue was excised, and serum was collected. Plasma protein levels were determined with a mouse insulin ELISA kit (ALPCO Diagnostics) and mouse adiponectin ELISA kit (R&D Systems). Whole mounts of the injected adipose pads were analyzed for expression of GFP with an Axiovert 200 inverted microscope (Ziess).

SUPPLEMENTAL DATA

Supplemental Data include four figures and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/135/2/240/DC1/.

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